METHOD FOR PRODUCING A TRANSGENIC PLANT WITH THE AID OF AGROBACTERIUM THUMEFACIENS

Field of the Invention

The present invention relates to the genetic engineering of plants and can be used for producing transgenic plants with desired properties.

Description of the Prior Art

The main damage to farm crops is caused by viral, bacterial and fungal pathogens, this damage materially exceeding that caused by insects and other pests taken together. In their turn, pathogenic fungi hold the first place in their harmfulness among other pathogens.

For example, effective cultivation of strawberry is limited by several diseases: red stele (caused by (*Phytophthora fragariae*), verticillaceous wilt (*Verticillium alboatrum*), leaf spot (*Mycosphaerella fragariae*), leaf blight (*Diplocarpon aerliana*), leaf roll (*Dendrophoma obscurans*), grey mold (*Botrytis cinerea*), powdery mildew (*Sphaerotheca humuili*) and others. Fungi which cause plant diseases not only affect the growth and development of plant organism, but often lower the productivity of cultivated plants.

In present-day agriculture effective control of fungal pathogens is achieved by a comprehensive approach. On the one hand, conventional selection methods are used to develop new varieties which display an enhanced resistance to definite races of fungi. But the development of one variety in such a way takes on an average abut 10 years. During this period of time pathogens often manage to mutate, and the new variety loses resistance and rapidly becomes obsolete. An alternative approach is offered by genetic engineering. A method of genetic transformation of plants makes it possible to transfer into plants separate disease resistance genes and obtain during a short period of time -plants with enhanced resistance to a wide range of phytopathogens.

Producing transgenic plants with an enhanced resistance usually encounters the problems of reproducibility of the known methods and the problems of producing plants with a low level of somaclonal variability.

Publications are known, devoted to optimizing the method of transformation of garden strawberry, developed by James et al (1990) and Nehra et al. (1990). In the method of transformation there were varied: 1) the composition of -the mineral and/or hormonal medium for the regeneration and selection of transformants, 2) the type and concentration of the selective agent, 3) the genotype of the variety, 4) the type of the strain and the vector construct, 5) the type and concentration of carbohydrates, 6) the type of the explant (leaves, petiole, tendril segments, meristem).

Du Plessis et al. (1999) have modified the method of agrobacterial transformation by substituting 6-benzylaminopurine in the selection and transformation medium by the synthetic growth regulator tidiazuron. It proved to be a more effective growth regulator for strawberry. Instead of the agrobacterial strain LBA4404 the authors began to use C58/PGW2260. The developed method suffers from a serious disadvantage: a very high frequency of somaclonal variations among transformants.

Dier et al. (2001) have shown that the substitution of sucrose by glucose in the media for the multiplication, co-cultivation, selection and regeneration of transformants essentially increases the frequency of regeneration of transgenic shoots. A solution is known, in which the authors make an attempt to decrease the frequency of somaclonal variations by substituting sucrose by maltose (Stuart et al, US Patent 4,801,545, 1989).

Mathews et al. (1998) have shown that the concentration and type of the selective agent in the culture medium have a very significant effect on the process of regeneration of transgenic shoots. The duration of cultivation on a selective medium is also of importance. Modification of the selection system made it possible to produce pure, non-chimeric lines consisting of transgenic cells only.

Dolgov et al. (1999), using the method described in James et al. (1990) and Nehra et al. (1990), developed for the Redcoat variety, produced a transgenic plant of garden strawberry of the Feyerverk variety. The effectiveness of the transformation method for the Fireworks variety turned out to be less than 1%, while for the Redcoat variety the effectiveness of the developed method was 6.5%.

De Mesa et al. (2000) have modified the method of genetic transformation by combining the method developed by James et al. (1990) and Nehra et al. (1990) with a ballistic method. Physical damage to leaf disks was made by the microprojectile bombardment method.

In the above-cited methods the preparation of explants is based on a single-step procedure of -preparing the whole amount of explants (James et al., 1990 and Nehra et al.,

1990). A method is known, in which as explants use is made of whole laminas on which a maximum number of mechanical injuries is inflicted simultaneously (Trinh et al., 2000). Both of these methods increase the frequency of necrosis.

Humara et al, (1999) have shown that inflicting ultrasonic microtraumas on tender tissue of pine cotgyledonary explants is accompanied by a lower frequency of necrosis. This method is applicable only for the embryonal tissues of germs or for any other cases when the regeneration proceeds from epithelial cells.

Publications are known, in which the method of genetic transformation is optimized for preventing necrotic reactions during the preparation of plant explants and their inoculation in an agrobacterial suspension.

Perl et al. (1996) tried to lower the frequency of necrosis by using antioxidants. Adding to the co-cultivation medium such components as DTT and PVPP made it possible to inhibit necrosis completely and succeed in producing fertile transgenic plants of grapes. However, the method is reproducible only on the plant material of one variety Superior Seedless.

Olhoft et al. (2001) have found that compounds with thiol groups, e.g., L-cysteine, are capable of increasing the frequency of transformation of *Glycine max* cells by agrobacteria. The effect manifests itself only in the stage of co-cultivation of explants with agrobacteria. The authors have also found that not only L-cysteine on adding to the co-cultivation medium can positively influence the process of transformation. A similar activity was displayed by such substances as glutathione, DTT, -sodium thiosulfate, copper ions and iron chelates. The above-cited substances inhibit the activity of plant polyphenol oxidases and peroxidases, suppressing thereby the development of necrotic reactions caused by wounding or pathogens.

However, all the cited chemical components added to the nutrient medium not only influence the process of the interaction of -bacteria with plant cells, but also produce a negative effect on the plant tissue of explants as such.

A combination approach for the elimination of necrosis is known (Hansen, US Patent 6162965). According to this method, in the stage of agrobacterial transformation chemical necrosis inhibitors are used, or an agrobacterium strain is selected which does not induce pronounced necrosis, or a genetic approach is used, in which, together with the gene of interest, the gene of specific necrosis-inhibiting factor is transferred into plant cells. Such factors have been known long since, but they have an extremely narrow spe-

cies-specific activity. The author presents no data about the influence produced by the lowering of necrosis on lowering the overall level of somaclonal variations.

The known modifications of the methods of transformation influenced most often the frequency of the regeneration of transgenic shoots or the frequency of transgenic callus formation. None of the improved methods led to considerable lowering of somaclonal variations and morphological changes of transgenic plants with an enhanced resistance to phytopathogenic fungi. For example, in the expression of thaumatin in transgenic plants of cucumber (Szwacka et al., 20002) an enhancement of resistance to *Pseudoperonospora cubensis* was detected only in part of transgenic plants, and the recombinant protein -expression level did not correlate with the resistance level.

There is known a large number of plant proteins, which are to one extent of another toxic for phytopathogens and can be used in producing plants resistant to pathogenic fungi. This is, first of all, a vast group of RP-proteins (pathogenesis-related proteins), comprising five families PR-1—PR5 (Linthorst 1991). To these proteins there also belong antimicrobial peptides (thionines, defensines and lectins) and ribosome-inactivating proteins. The antifungal activity has been studied best of all in proteins belonging to two families PR-3 (glucanases) and PR-4 (chitinases). Both types of proteins belong in terms of the activity mechanism to hydrolyzing enzymes which destroy the structural components of the cytoderm of fungi.

The PR-5 family or thaumatin-like proteins in this aspect have been much less studied. They can display antifungal activity against a wide range of pathogenic fungi (Abad et al, 1996), e.g., such as *Alternaria solani, Aserrgillus flavus, Aspergillus parasitica, Bipolaris maydis, Bipolaris zeicola, Phytophthora fragariae, Verticillium alboatrum, Mycosphaerella fragariae, Diplocarponearlianas, Dendrophoma obscurans, Botrytis cinerea, Sphaerotheca humuli, Fusarium graminiarum, Fusarium oxysporum,* and others. There are no unambiguous communications about the antibacterial activity of thaumatin-like proteins in transgenic plants. It is known that, like the rest of the PR protein groups, they, apparently, perform protective function in plant cells. For instance, it is known that the expression of RS-5 (TL) proteins in plants is activated by attacks of pathogens, mechanical injuries, and also by such metabolites as salicylic acid and ABA (Stintzi et al. 1993). It is also known that such thaumatin-like protein from maize as zeamatin displays antifungal activity *in vitro*, but only in combination with the antibiotic Nikkomycin Z which inhibits the synthesis of chitin (Roberts et al. (1990). Hence, zeamatin behaves as

a co-factor enhancing the action of the sublethal concentration of the antibiotic. Under the same conditions thaumatin also displays antifungal activity

Dolgov et al. (1999) transferred thaumatin gene into plants of apple, carrot. pear and strawberry. The authors have confirmed only the integration of the gene into the genome of the plants, but the protein expression in transgenic tissues was not analyzed.

Schestibratov et al (2001), by using the known method of genetic transformation (Jamers et al. 1990 and -Nehra et al. 1990)-, have produced plants of garden strawberry with the gene of thaumatin. The recombinant protein expression was confirmed only in several transgenic lines.

None of the known methods provides the possibility for producing transgenic plants, say, of garden strawberry, with expression of thaumatin-like proteins and, particularly, of thaumatin, which display an enhanced resistance to phytopathogens and have a low level of somaclonal variations.

Known in the art is US Patent 5,856,154 (Ryals et al. 1999) for a method of protecting plants from pathogens, which provides the production of chimeric genes encoding PR-1 proteins and is based on using chemical agents for setting up conditions for the development of systemic acquired resistance (SAR). The authors select the types of genetic constructs with taking into account the use of facts relevant to the systemic acquired resistance of plants, which is yielded by plants in response to the influence of external factors.

Also known is a method of producing transgenic plants by introducing into a plant two genes producing an SAR effect (Baker et al., US Patent Application 20020004944, 2002). Nevertheless, in these publications no data can be found which would support the fact that plants being transformed eventually show not only resistance, but also preserve all morphological characteristics.

Disclosure of the Invention

One of the subjects of the invention is an improved method of agrobacterial transformation and regeneration of transgenic plants, which is characterized by low frequency of the necrosis of explants, enhanced frequency of transient expression, enhanced frequency of the regeneration of transgenic shoots, higher proportion of direct transformants, owing to the formation of acquired resistance to abiotic and biotic stresses in leaf disks, which eventually leads to lowering the frequency of somaclonal variations in the transgenic plant.

According to an improved method, the stages of the preparation, inoculation and co-cultivation of explants comprise the following steps:

i) a step of selecting one or more leaf segments for preparing explants; ii) a step of preparing leaf disks by separating a segment from each disk, followed by inoculating and co-cultivating leaf disks with agrobacteria; iii) a step of removing excess agrobacteria from leaf disks, separating a first lot of explants from the side of the first section; iv) a step of transferring explants onto the selection and regeneration medium; v) a step of preparing subsequent lots of explants in accordance with steps iii) and iv) till the last lot of explants from the selected leaf disks has been formed; wherein the preparation of each of the subsequent lots of explants is carried out after a time interval required for the transformation of plant cells and formation of acquired resistance to abiotic and biotic stresses in the leaf disks.

A next subject of the invention is a vector construct which contains genetic material coding for at least one peptide belonging to the group of target proteins and/or proteins responsible for enhancing the resistance to phytopathogens and/or for lowering necrosis.

Another aspect of the invention is that genetic material codes for the resistance of a transgenic plant to fungi selected from the group: *Phytophthora fragariae, Verticillium alboatrrum, Mycospaerella fragariae,Diplocarpon earliana, Dendxrophoma obscurans, Botrytis cinerea, Sphaerotheca humuli.*

A further subject of the invention is a method of producing a transgenic plant which enters into the group of dicotgyledonous plants: apple, pear, garden strawberry, carrot, and tomatoes.

A still further subject of the invention is a method of producing a transgenic plant of garden strawberry, selected from the group of varieties: Selekta, Chambly, Chandler, Oka, Yamaska, L'Acadie, L'Authentique Orleans, Rosalyne, Roseberry, Saint-Pierre, Donna, Enzed Levin, Enzed Lincoln, Vilanova, Durval, Redcrest, Bountiful, Redgem, Pelican, Primtime, Mohawk, Latestar, Winoma, Feyerverk.

Brief Description of the Drawings

Fig. 1A shows diagrammatically plasmid pUR528;

Fig. 1B shows diagrammatically plasmid pBBThau;

Fig. 1C -shows a restriction fragment XbaI-BamHI from plasmid pBBThau carrying preprothaumatin II;

Fig. 2 shows diagrammatically binary vector pBITau35. LB and RB are left-hand and right-hand terminal repeats of T-DNA; Panos is a terminator from the gene of nopalin synthetase; pNOS is a promoter from the gene of nopalin synthetase; p35S is a promoter of 35S RNA of cauliflower mosaic virus; nptII is a gene of the plant selective marker of neomycin phosphotransferase; thauII is preprothaumatin II sequence coding for super-sweet protein thaumatin II;

Fig. 3 -illustrates the influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the frequency of necrosis in the tissues of explants of garden strawberry of the Feyerverk variety;

Fig. 4 illustrates the influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the transient GUS-expression in the tissues of explants of garden strawberry of the Feyerverk variety;

Fig. 5 illustrates the influence of stagewise co-cultivation on the frequent cy of formation of tissues resistant to kanamycin (Km+) on the starting explants of garden strawberry of the Feyerverk variety;

Fig. 6 illustrates the influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the frequency of regeneration of transgenic shoots of garden strawberry of the Feyerverk variety;

Fig. 7 illustrates the influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the transgenic GUS-expression in the tissues of explants of apple.

Variants of Carrying out the Invention

An improved method of producing transgenic plants on the examples of garden strawberry and apple is based on the use of natural molecular mechanisms which are activated in plants in the process of the development of locally acquired resistance to abiotic and biotic stresses.

A method of producing transgenic plants with improved agronomic characteristics comprises the following steps.

In the first step the properties of a transgenic plant are selected and vector constructs for transferring the required genetic material into plant cells are produced.

In the second step the variety (genotype) is selected, stools are prepared for their subsequent sterilization and introducing into the culture *in vitro*, microclonal reproduction of the plant material is carried out, and rooted plants are prepared *in vitro*, which will serve as a source of leaf explants.

In the third step agrobacterial transformation is carried out with the use of constructed vector plasmids. For this purpose a bacterial suspension is prepared, leaves are collected and prepared, a stagewise inoculation and co-cultivation of the explants are carried out in accordance with the improved method.

In the next step selection of transgenic tissue, selection of transformants and elimination of -agrobacteria are carried out and microclonal reproduction of the selected transformants is effected.

Transgenic plants having been produced, a molecular-genetic analysis of transgenic lines is carried out. For this purpose a total DNA is isolated from the tissue of the transgenic lines, PCR analysis of the heterologous DNA integration and histochemical analysis of the GUS-activity are carried out, along with Western-blotting for analyzing the expression of the target gene in the leaves and fruits of the transgenic lines.

In the final step, for selecting lines without somaclonal variations, biological testing is carried out: -an analysis of resistance to phytopathogens; an organoleptic analysis of fruits; evaluation of the vegetative and generative activities of the transgenic plants in field conditions.

In the improved method of transformation the step of preparing, inoculating and co-cultivating explants with agrobacteria is carried out stagewise in the following manner.

Explants from the same leaves are cut not at the same time, before the inoculation, but in at least in more than two independent approaches with a periodicity of 1 to 5 days, more preferably in 3 days. The number of steps is selected in the range of from 2 to 5r, preferably from 3 to 4.

The explant width is selected within 0.5 to 10 mm, preferably within 1 to 3 mm. The ratio of the section length and the explant surface area is selected within 0.1 to 2 mm/mm^2, the more preferable value being 0.5 mm/mm^2.

For enhancing the transformation effectiveness, growth regulators and/or selective agents are comprised in the formulation of the selection and regeneration medium. Cytokinins and/or auxins are used as the growth regulators. For example, synthetic derivatives of phenylurea can be used as cytokinins, and IBA, IAA, PAA, 2,4-D and their conjugates with amino acids can be used as auxins. Nevertheless, for enhancing the acquired resistance to abiotic and biotic stresses, growth regulators can be excluded from the formulation of the co-cultivation medium. The selective agent is selected from the group comprising: neomycin, kanamycin, hygromycin, phosphinotricin. The combination, ratio and concentration of said components are selected depending on the genotype.

For instance, in the case of transformation of garden strawberry, the TDZ concentration is selected within the range of from 1 to 10 mg/ml. The optimal TDZ concentration is 5 mg/l. The TBA concentration is selected within the range of from 0 to 2 mg/l. The optimal TBA concentration is 0.3 mg/l. The kanamycin concentration is selected within the range of from 10 to 100 mg/l. The optimal kanamycin concentration is 50 mg/l.

For producing a transgenic plant with prescribed properties vector constructs are used, produced by the known method (Maniatis T. et al. 1982), which comprise a genetic material selected from the group consisting of: a) a genetic material coding for at least one target protein; b) a genetic material coding for at least one protein which is instrumental in lowering necrosis at the step of transformation; c) a genetic material coding for at least one protein which enhances the plant resistance to phytopathogens and which is selected from the group: PR-1, PR-2, PR-3, PR-4, PR-5; d) a genetic material whose coding sequence may consist of at least two sequences related to sub-items a) and b), ort a) and c), or b) and c), or a), b), c).

For producing a plant with enhanced resistance to pathogens, the genetic construct comprises genes coding for the resistance to fungi e selected from the group consisting of *Phytophthora fragariae, Verticillium alboatrum, Myucosphaerella fragariae,Diplocarpon earliana, Derndrophoma obscurans, Botrytis cinerea, Sphaerotheca humuli.*

For instance, for producing a transgenic plant of garden strawberry with enhanced resistance to *Botrytis cinerea*, vector construct pBITau35 is used, which comprises the thaumatin II gene (Schestibratov et al. 2002).

As a result of genetic transformation, plants are produced, which contain genetic material coding for at least one protein entering into the group of target proteins and/or proteins responsible for the enhancement of resistance to phytopathogens and/or for lowering of necrosis.

As the target product use can be made of proteins, which : a) enhance the nutritive value, b) improve the taste, c) impart resistance to herbicides, d) are of pharmaceutical importance.

The improved method of genetic transformation can be used for monocotyledonous and dicotyledonous plants.

For producing transgenic plants with enhanced nutritive value, improved taste, and enhanced resistance to phytopathogens dicotyledonous plants can be selected.

For example, for the transformation plants are used, selected from the group: apple, pear, garden strawberry, carrot and tomatoes.

Transgenic plants of garden strawberry can be produced, selected from the group of varieties: Selekta, Chambly, Chandler, Oka, Yamaska, L'Acadie, L'Authentique Orleans, Rosalyne, Roseberry, Saint-Pierre, Donna, Enzed Levin, Enzed Lincoln, Vilanova, Durval, Redcrest, Bountiful, Redgem, Pelican, Primtime, Mohawk, Latestar, Winoma, Feyerverk.

Examples in a detailed description are given for garden strawberry of Feyerverk and Selekta varieties and for apple of Melba variety.

For practical application transgenic plant components can be used: transgenic cells, organs (leaves, stalks, roots, flowers), whole plants, seeds and fruits.

Modifications introduced into the method of agrobacterial transfer make it possible to make the following improvements in the parameters of the method as such and in the characteristics of transgenic plants. The method makes it possible: to lower the frequency of development of necrotic reactions on the explants being transformed (Fig. 3); to raise the frequency of transient expression of the genes being introduced (Fig. 4); to raise the frequency of formation of new transgenic tissues on the initial explants (Fig. 5); to increase the ratios of direct regenerants to the number of regenerants produced via the callus stage (Table 6); raise the frequency of regeneration of transgenic shoots (Fig. 6); finally, to increase the proportion of plants without somaclonal variations among transgenic lines (Table 11).

Experimental Protocols

These experimental protocols relate to the methods, conditions and components of media for the procedure of producing transgenic plants with an enhanced resistance to phytopathogens and improved taste characteristics on the example of garden strawberry. These protocols include but do not limit changes in the concentration parameters, time-related conditions and other changes which are obvious from the general state of the art and come into the scope of protection of the present invention.

A. Materials and methods used in producing a transgenic plant .Constructing a vector plasmid for transferring into plant cells *preprothaumatin II* sequence encoding thaumatin II protein.

The thaumatin II gene encodes the super-sweet protein which is encountered in nature in fruits of *Thaumatococcus dantelli* from which it was isolated and characterized. The sequence comprises *preprothaumatin II* — a precursor of mature thaumatin, which contains a native signal peptide. Cloning of thaumatin cDNA is described in Edens et al;,

1982. The use for the expression in cells of *E.coli* is shown by Verrips et al (US Patent 4,891,316).

The vector plasmid for transferring into plant cells of the *preprothaumatin II* sequence, encoding thaumatin II protein, was constructed with the use of well-known molecular cloning methods (Sambrook et al., 1989).

Plasmid pBI121 (Clontech Co.) was taken as a basis for transferring -the given gene into plant cells. The sequence of the gene *gus* in this plasmid was changed by the *preprothaumatin II* fragment from plasmid pUR528 (Unilever Research Laboratorium, The Netherlands; Erdens et al., 1982; (Fig. 1A). the *preprothaumatinII* fragment from the plasmid `pUR528 was excised with respect to EcoRI and HindIII sites. The intermediate vector pBB (selective marker Ap) carrying the polylinker SphI-XbaI-XhoI-EcoRI-SmaI-AvaI-BamHI-Xbal-SDauI-PstI-HindIII-BamHI-BstXI-NciI was incubated with EcoRI and HindIII restrictases. The *preprothaumatin II* fragment with sticky ends along the EcoRI and HindIII sites was ligated into linearized pBB vector along the same sites. The obtained pBBThau plasmid (Fig. 1B) was incubated with the XbaI and BamHI, with as view to cutting out again the *preprothaumatin II* fragment, but this time with new sticky ends (Fig. 1C). Binary vector pBI121 was first incubated with SmaI and SacI restrictases, then the linearized form of the plasmid was ligated along blunt ends, then the preparation was incubated with the XbaI and BamHI restrictases. TheXBaI-BamHI fragment of the *preprothaumatin II* sequence, preliminarily excised from the pBBThau vector, was ligated into the linearized pBI121 vector. The pBIThau vector is shown diagrammatically in Fig. 2.

Media for cultivating garden strawberry *in vitro* and carrying out genetic transformation. Media for the multiplication (M) elongation and rooting (ER), co-cultivation (CC) and regeneration of transformants (RT) are prepared in accordance with the data presented in Table 1. In all the media pH is adjusted to 5.5.

Table 1. Media for cultivation of garden strawberry *in vitro* and carrying out genetic transformation

Components	Concentration			
	M medium	ER medium	CC medium	RT medium
Potassium nitrate	316 mg/l	316 mg/l	2900 mg/l	2900 mg/l
Ammonium nitrate	400 mg/l	—	1650 mg/l	1650 mg/l
Monosubstituted potassium phosphate	170 mg/l	170 mg/l	170 mg/l	170 mg/l
Calcium nitrate × $4H_2O$	1133 mg/l	1133 mg/l	—	—
Calcium chloride	—	—	332.2 mg/l	332.2 mg/l
Magnesium sulfate × $7H_2O$	369 mg/l	369 mg/l	180.7 mg/l	180.7 mg/l
MS microsalts	32.83 mg/l	32.83 mg/l	32.83 mg/l	32.83 mg/l
Iron sulfate × $7H_2O$	27.8 mg/l	27.8 mg/l	27.8 mg/l	27.8 mg/l
Na_2EDTA × $2H_2O$	37.26 mg/l	37.26 mg/l	37.26 mg/l	37.26 mg/l
Sucrose	30 g/l	20 g/l	30 g/l	30 g/l
MS vitamins	3.5 mg/l	3.5 mg/l	3.5 mg/l	3.5 mg/l
Myo-inositol	100 mg/l	100 mg/l	100 mg/l	100 mg/l
Agar	8 g/l	8 g/l	8 g/l	8 g/l
BAP	1 mg/l	—	—	4 mg/l
TDZ	—	—	—	1 mg/l
IBA	—	—	—	0.3 mg/l

Selection of variety and preparation of stock plants

In the experiments on genetic transformation of strawberry, *in vitro* material of the following plants was used: a) of the Feyerverk variety (Zubov A.A., VNII Genetiki I Selektsii Plodovykh Rastenij im. I.V. Michurina, Michurinsk) and b) of the Selekta variety (Evans E., South Africa, 1973, **PI** 551873).

Garden strawberry was introduced into the culture *in vitro* by sterilizing young apical buds from the tendrils of open ground plants. For this purpose tip buds were cut off with a small shoot fragment no bigger than 5—10 mm, then cleared from surface leaflets, soaked in an aqueous solution of Tween 20 for 1 hour, after that washed under running water for 2 hours. For sterilizing, washed explants were kept for 1—5 minutes, depending on their size and age, in a 2% solution of sodium hypochlorite. After that the explants were washed thrice in 100 ml of sterile distilled water.

The surface-sterilized shoots were planted separately into test tubes with 10 ml of the medium for multiplication (M medium, see Table 1). The main components of this medium correspond to the MS medium (Murashige et al. 1962) improved according to Boxus (1974), only with two modifications: first, glucose in the concentration of 22 g/l was replaced by 30 g/l of sucrose; second, for stimulating the multiplication, the medium was complemented with 5 Mm of ammonium nitrate. After keeping for one month in the culture, whole uninfected explants with newly formed shoots were replanted onto the elongation and rooting medium (ER medium, se Table 1), which differs from the M medium by the absence of growth regulators, additional ammonium nitrate and lowered sucrose concentration (2%). On an average in 4—6 weeks the grown up shoots are separated from one another and planted onto a fresh medium having the same formulation, to stimulate rooting. The rooted plants were used as the source of leaf disks -in subsequent regeneration and transformation experiments. Such plants are cultivated on the ER medium for 7—8 months, with replanting every month onto a fresh medium. For growing sterile material of strawberry, -special 250 ml screw-cap cultivating jars (HortMic, Finland) were used.

Preparation of bacterial suspension

For the genetic transformation with binary strains pBI121 and pBIThau35, supervirulent strain *Agrobacterium thumefaciens* CBE21 (Revenkova et al. 1994) was used, constructed on the basis of wild strain *A. thumefaciens* with Ti-plasmid pTiBo542.

Bacterial suspensions of the strains CBE21/pBITThau35 and CBE21/pBI121 for the inoculation of the explants were built up overnight in 50 ml of the LB medium (Table 2) at 28°C. Before the inoculation, the cell suspension was centrifugated at 5000 rpm for 5 minutes. The precipitate was washed twice with 50 ml of liquid MS medium to remove residues of the LB medium. The washed cell residue was re-suspended in liquid MS medium, then the suspension density was brought to **OII**600 equal to 1.

Table 2. LB medium for cultivation of agrobacteria

Component	Concentration
Sodium chloride	10 g/l
Bacto Trypton	10 g/l
Yeast extract	5 g/l
pH	7.5

Collecting and preparing leaves

Young fully unfolded trifoliums were cut off the stools prepared by the above-described method directly before setting up the genetic transformation. The size of a separate leaf disk in each trifolium varied within 10—15 mm (in a direction of the central vein). To reduce shrinkage of the leaves in the course of subsequent operations, they are kept in closed Petri dishes with a small quantity (20 ml) of liquid MS medium. Leaves are cut off the plants not oftener than twice a month. The stools before and after collecting the leaves are cultivated under the same conditions of a 16-hours' daylight period.

Preparation, inoculation and co-cultivation of explants

The cut-off trifoliums are separated into individual leaf disks which, in their turn, are cut with a scalpel into explants by various methods.

Improved method of preparing, inoculating and co-cultivating explants

In the proposed method leaf disks are cut with a scalpel on a Petri dish in the presence of a small quantity of liquid MS medium. From 10—15 mm leaf disk (in a direction of the central vein) a narrow strip having a width not exceeding 2 mm is cut off and then discarded The remaining larger part of the leaf disk (explant A-5) is used later for the inoculation.

In the first step explants A-5 immediately after cutting them off are transferred carefully with pincers into the bacterial suspension and soaked there for 30 minutes, Then explants A-5 are transferred into clean Petri dishes with paper filters and dried a little for 5 minutes to remove excess bacteria. After that these explants are placed into Petri dishes with the CC medium, the surface of which is covered with a paper filter. The dishes are wrapped with parafilm and incubated in a temperature-regulated chamber in darkness at a temperature of 25—28°C for 3 days.

Then explants A-5 are transferred into a beaker with liquid MS medium, and stirred intensively for 3—5 minutes to remove excess bacteria. The washed explants are transferred into Petri dishes with a small quantity of liquid MS medium. From each of the explants A-2 a narrow strip having a width of about 2 mm is cut off with a scalpel from the side of the first section, thus forming first-stage explants. Explants A-1 are collected into as beaker with liquid MS medium. After that explants A-1 are placed into Petri dishes onto the surface of RT medium to which 500 mg/l of cefotaxime are added, and intensively stirred to remove agrobacteria residues. After that explants A-1 are placed into Petri dishes onto the surface of RT medium with the rear side facing the medium. The dishes are wrapped into parafilm and incubated in a temperature-regulated chamber in

darkness at a temperature of 23—25°C. Explants A-5 are returned again onto the same CC medium, where the paper filter was preliminarily changed. The dishes are wrapped into parafilm and incubated in a temperature-regulated chamber in darkness at a temperature of 25—28°C for the next 3 days.

In the second step explants A-5 are transferred into a beaker with liquid MS medium, intensively stirred for 3—5 minutes to remove excess bacteria. The washed explants are transferred into Petri dishes with a small quantity of liquid MS medium. A next strip having a width of 1 to 2 mm is cut off with a scalpel from explants A-5 from the side of the first section, second-stage explants being thus formed. Explants A-2 are collected into a beaker with liquid MS medium to which 500 mg/l of cefotaxime are added, and intensively stirred to remove agrobacteria residues. After that explants A-2 are placed into Petri dishes onto the RT medium with the rear side facing the medium. The dishes are wrapped into parafilm and incubated in a temperature-regulated chamber in darkness at a temperature of 23—25°C. Explants A-5 are returned again onto the same CC medium, where the paper filter was preliminarily changed. The dishes are wrapped into parafilm and incubated in a temperature-regulated chamber in darkness at a temperature of 25—28°C for the next 3 days.

In the third step and in the fourth step from explants A-5 third-stage explants A-3 and fourth-stage explants A-4 are formed in accordance with the procedure described for the second step.

In the fifth step the last explant A-5 is treated, which is transferred into a beaker with liquid MS medium, intensively stirred for 3-5 minutes to remove excess bacteria. After that the explant is washed with liquid MS medium to which 500 mg/l of cefotaxime are added to remove agrobacteria residues. After that explants A-2 are placed into Petri dishes onto the RT medium with the rear side facing the medium. The dishes are wrapped into parafilm and incubated in a temperature-regulated chamber in darkness at a temperature of 23—25°C.

The known method of preparing, inoculating and co-cultivating explants (James et al., 1990) is used as control.

<u>Selection of transgenic tissue, selection of transformants and elimination of agrobacteria</u>

After the inoculation and co-cultivation with agrobacteria, explants are placed into Petri dishes onto the surface of RT medium complemented with 600 mg/l of casein hydrolyzate , 500 mg/l of cefotaxime and 50 mg/l of kanamycin. The dishes are wrapped into

parafilm, and incubated in a temperature-regulated in darkness at a temperature of 25—28°C. Cefotaxime is used for the elimination of agrobacteria residues on the explants. Its concentration is varied during the passages with every monthly passage, the level is lowered by 100 mg/l from the initial 500 mg/l to the final 0 mg/l. Kanamycin plays the role of selective agent, because in vector constructs the neomycin *nptII* transferase gene is used as the selective marker. The working concentration of kanamycin, sufficient for inhibiting the growth of the non-transgenic tissue of garden strawberry is 50 mg/l. However, for stimulating the regeneration of adventive shoots, the concentration of kanamycin in the medium is reduced by one half after separating callus pieces from the initial tissue and transferring them onto a fresh medium. Separating transgenic callus from the necrotic tissue of the explants is the crucial moment of the process of selection and regeneration of the transformants. The effectiveness of the transformation without this procedure lowers markedly.

The regeneration of the transformants of strawberry can proceed in two ways: directly from the cells of the explants and/or via the intermediate callus stage. In the first case transformants appear from the first through the second month of selection. Regeneration via the callus stage is appreciably extended in time, transformants may appear from the third through the sixth month of cultivation on the selective medium.

Multiplication of transformants

The shoots that have regenerated are separated from the initial tissue of the explants and transferred for the multiplication onto the M medium (Table 1), complemented with 0.1 mg/l of TDZ and 2.5 mg/l of kanamycin. After a one month's passage , the multiplied shoots are transferred in whole clusters onto the ER medium for stimulating the elongation of the shoots. After 4—5 weeks the grown up shoots are separated one from the other and planted separately on a fresh medium of the same formulation to stimulate rooting. Rooting proceeds during 4—6 weeks. The rooted plants are used later on for the adaptation to green-house conditions and subsequent molecular-genetic analysis and biological testing.

B. Materials and methods for analyzing transgenic plant properties

Isolation of total plant DNA of garden strawberry

For the extraction of genomic DNA use was made of both *in* vitro and *in* vivo plant material. From the *in vitro* conditions for the trituration in liquid nitrogen whole shoots cultivated on the medium for the multiplication were taken. From green-house plants young, yet unfolded leaflets were cut off. In both cases the age and physiological condition of the plant tissue strongly influenced the purity the purity of the preparations. For

sterile material the optimal age of the shoots was 3—4 weeks; for green-house material the age of the leaflets was not more than 5—6 days. Isolation was carried out according to the protocol modified by us. The procedure of Rogers et al. (1994) with the use of 2^x STAV buffer was adopted as the basis.

In contradistinction to the protocol according to Rogers et al. (1994), homogenized tissue is re-suspended in 1 ml of a washing buffer: 100 mM of potassium acetate, pH 4.5, 20 mM EDTA, 1% PVP, 1% 2-ME. The resulting suspension is centrifugated for 5 minutes at 4.500 rpm. The supernatant liquid is removed. The precipitate is again re-suspended in 600 µl of an extraction STAV buffer having the following composition: 100 mM of tris-HCl, pH 8, 2.5 M NaCl, 20 mM of EDTA, 2% STAV, 40 mM of 2-ME.

PCR analysis of transgenic lines

The lines prepared with the aid of any of the employed vector constructs are analyzed with two pairs of primers: for the insertion of selective marker (*nptII*) and for the insertion of sense gene (*thauII*) or reporter gene (*uidA*).

The PCR analysis of the integration of different heterologous sequences into the genome of garden strawberry is carried out in a reaction medium which contains: 67 mM of Tris-HCl, pH 9.0, 16 mM of $(NH_4)_2SO_4$, 2 mM of $MgCl_2$, 0.01% gelatin, 200 µM of each dNTP. The concentration of the primers and of the polymerase and the temperature conditions are selected for each particular case.

For the amplification of the of the gene *nptII* fragment, primers to 0.6 mM final concentration and 0.05 U/µl of Taq polymerase are introduced into the reaction mixture. Amplification conditions: 5 minutes of denaturation at 94°C (hot start), 30 seconds of denaturation at 93°C, annealing — 45 seconds at 62°C, elongation — 45 seconds at72°C, 30 amplification cycles. The expected size of the fragment being amplified is 742 nucleotides.

The insertion of *thauII* was determined under conditions for the most part similar with those for *nptII*, only the concentration of the primers was 0.9 µM each, 0.1 U/µl of the polymerase, and the temperature conditions were changed as follows: hot start, 2 min 94°C; denaturation, 30 s 93°C; annealing, 40 s 63°C; elongation, 35s 72°C; 35 amplification cycles. The expected size of the fragment being amplified is 878 nucleotides (Schestibratov et al., 2002).

Histochemical analysis of GUS activity

Histochemical determination of GUS activity was carried out by the method of Jefferson (1987). The histochemical determination of the GUS activity was carried out with the use of 5-bromo-4-chloro-3-indolyl glucoronide (X-GLUC, Duchefa). For the determination, plant tissue was placed into a buffer: 50 mM of $NaPO_4$, pH 7.0, 10 mM of Na_2EDTA, 0.12% Triton X-100 containing 1 mg/l of X-GLUC, incubated for 6 hours at 37°C. After that the tissue was washed several times with 50% ethanol, and stained tissues were stored at 4°C in 70% ethanol.

Organoleptic analysis of garden strawberry fruits

Fruits for the organoleptic analysis were gathered from transgenic plants cultivated in field conditions on the certified site for testing transgenic cultures on the territory of the VNIISPK quarantine garden (at Orel). Fruits of the first yields of the first and second years of cultivation were tasted. Fruits of 12 independent transgenic lines and control lines were evaluated according to their taste qualities, first of all, by sweetness. The sweetness of the fruit pulp was evaluated in terms of a five-point system, proceeding from the Feyerverk variety characteristic, according to which the taste of middle-ripening fruits of the first yield does not exceed 3.8 to 4.0 points.

In connection with the specific properties of thaumatin which is responsible for long-term aftertaste, the organoleptic analysis was carried out pairwise. For this purpose fruits of two different plants were taken and pairs were formed: a) line with thaumatin expression — line without thaumatin expression; b) line with thaumatin expression — non-transgenic plant.

Combinations were made up arbitrarily, with taking into account only the data about the presence or absence of protein. After tasting each sample, tasters gargled their oral cavity with distilled water. In order to rule out or at least decrease the effect of differences in ripeness on the correctness of the analysis, fruits of the same size and color were chosen for tasting. The weight of the fruits varied from 10 to 15 grams. One taster for comparison for each line was given four sections, each from different fruits. The tasters allotted independent points, the arithmetic mean serving as the taste rating for the given specimen.

Analysis of the influence of thaumatin II expression on antifungal resistance of garden strawberry to *Botrytis cinerea*

The culture of the phytopathogen *Botrytis cinerea* is isolated from infected fruits of garden strawberry of the Feyerverk variety. Repeated re-inoculation of uninfected fruits and leaves confirmed the virulence of the isolated and purified pathogen. The culture of

the pathogen under *in vitro* conditions was maintained on potato medium PDA, in darkness at 28°C. For preparing this medium, a decoction of pealed potato in tap water was used (200 g of potato per liter of water cooked for 30 minutes after boil). 10 g/l of glucose and 20 g/l of bacteriological agar were added to the decoction. The medium was autoclaved under pressure of 1 atm and at 120°C for 20 minutes. After autoclaving, casein hydrolyzate was added to the medium to the final concentration of 300 mg/l.

For preparing a suspension of spores, a 7—8 days' culture of the fungus was used. After passage on a fresh medium, during a week new mycelium develops from the spores, and sporogenesis starts on the 7^{th} to 8^{th} day. Spores were gathered with a sterile spatula which was each time rinsed in a solution of 25 mM sodium acetate (pH 5.2) with 5% glucose and a detergent (2 drops of Triton X100 per 20 ml). The density of the suspension for all the bioassays was brought to $2—3\times10^6$ spore/ml. Density counts were carried out in a Goryaev chamber.

Transgenic plants produced with the aid of the vector construct pBIThau35 were analyzed for resistance to the phytopathogen *Botrytis cinerea* in accordance with the method described in Peng et al. 1991 with additional improvements which are described below.. Material for the bioassay was taken from green-house plants not older than 4—6 months. Before the inoculation, leaves were slightly died at room temperature. Inoculation was carried out by wetting leaves with the suspension of spores for 1—2 minutes. The inoculated leaves were placed into Petri dishes onto moist filters and incubated during 24 hours in darkness at 28°C. Then the leaves were sterilized in a 0.5% solution of sodium hypochlorite for 2 minutes. The sterilizing agent was washed away thrice with 100 ml of sterile distilled water. Leaf disks were cut from the leaves with a cork drill of 7 mm in diameter. 10 disks were made from each trifolium. In contradistinction to the known method, the disks were placed onto moistened filters rather than onto an agarized medium with additions of paraquate and chloramphenicol.

The experimental and control materials were compared pairwise within one Petri dish to avoid the influence of humidity fluctuations of the filters and air inside the chamber. The already cut and placed leaf disks were incubated under the same conditions as the whole leaves. On the tenth day the development of the infection was assessed on the basis of three criteria: 1) the degree of development of the surfaced mycelium; 2) the area of the necrotic lesion of the leaf disks; 3) the degree of sporogenesis. The first and third criteria

were evaluated in points from 0 to 5. The degree of necrosis was evaluated in the percentage of the area of the affected part of the disk.

Field tests of transgenic plants of garden strawberry with the gene of super-sweet protein thaumatin II

A large part of the transgenic plants produced with the help of the vector pBIThau35 was transferred to the VNIISPK quarantine garden (at Orel) for planting in open ground and carrying out field tests.

When planting the transgenic lines and control plants, randomization was carried out. The plants intended for planting, (5 to 10 for a separate line) were distributed into three groups, each of which was planted in different locations of a certified plot.

Four new rosettes from formed tendrils were rooted from each stool plant. For the planting not to be crowded, the rest of the tendrils were regularly removed. Before the onset of flowering, the plot was covered with a protecting material for preventing the propagation of the pollen of the transgenic plants. An individual cover of about 60 cm in height was erected above each of the three rows. After gathering the first yield of the fruits, the vegetative and productive activities of the planted plants were evaluated. The number of rosettes in a shrub, the number of peduncles and an average height of the shrub were counted. One month after the gathering of the second yield, the fruits of the first and second yields were used for analyzing the thaumatin expression by the method of Western blotting, and also for organoleptic evaluation of the effect produced by heterologous protein on the taste of the fruits.

Examples

Example 1. The influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the frequency of necrosis in the tissues of explants of garden strawberry of the Feuerverk variety

The frequency of necrosis of the explants was evaluated visually after 15 days of cultivation on the selective medium. The plant material transformed by following the standard and improved methods was compared. The obtained data suggest that the improvement of the inoculation and co-cultivation stages tells positively on the survival of the explants and on preventing the development of necrotic reactions in the places of wounding. The data on the average frequency of the necrosis of the explants transformed according to the improved method (A), 16.6%, and according to the known method (B), 54.8%, show

that the necrosis of the tissues was lowered almost by the factor of 3.5. The data are presented in Fig. 3.

Example 2. The influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on transient GUS expression in the tissues of explants of garden strawberry of the Feyerverk variety

In the genetic transformation with the use of *Agrobacterium thumefaciens*, T-DNA with the required heterologous sequences is transferred into plant cells predominantly in the wounded sites, where direct contact of the bacteria with the injured cells is ensured. For evaluating the effectiveness of the proposed method, histochemical analysis of the GUS activity in the tissues of the explants was carried out on completion of the co-cultivation period. Such analysis makes it possible the frequency of the transfer and expression of the recombinant genes in the T-DNA composition from the bacteria into the plant cells. The plant material for the analysis is selected on expiration of the 3-days' period of co-cultivation, immediately after washing-off the explants from the bacteria. Histochemical staining of the explants is carried out for 7 hours at 37°C in an X-Gluc solution for the histochemical analysis of the GUS activity. The results of the analysis are presented in Fig. 4.

Example 3. The influence of stagewise co-cultivation with *Agrobacterium thumerfaciens* on the frequency of formation of kanamycin-resistant tissues (Km+) on the initial explants of garden strawberry of the Feyerverk variety

In subsequent co-cultivation (after their stagewise co-cultivation with agrobacteria) on the RT medium complemented with 500 mg/l of cefotaxime and 50 mg/l of kanamycin, during first two monthly passages on the explants there takes place formation of direct transformants and non-organized callus groups. Km+ tissues (regenerants and callus groups) are formed predominantly on the sites of wounding.

By the end of the second passage the proportion of the explants on which transgenic tissue was formed, was calculated. From stage A-1 through stage A-4 a stable growth of the frequency of formation of Km+ tissues is observed. Apparently, this is associated, in the first place, with a decrease in the intensity of necrotic reactions in response to wounding and to the subsequent co-cultivation with agrobacteria; in the second place, with possible increase of the morphogenetic potential. In stage A-4 maximum proportion of the explants which have formed transgenic tissues was registered (89.5%, see Fig. 5). A reduction of the proportion of the explants which have formed transgenic tissues in group A-5 (54.9%) is explained by that in the fifth stage the number of plant cells competent for

the genetic transformation already lowers. In control group B transgenic tissue formed on 27.1% of the explants, this being in good correlation with the high frequency of necrosis (54.8%, see Example 1).

Example 4. The influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the frequency of regeneration of transgenic shoots of garden strawberry of the Feuerverk variety

The frequency of the regeneration of transgenic shoots is one of the main characteristics of the efficiency of the method of genetic transformation. This efficiency is composed of the efficiencies of separate stages, starting with the preparation of explants and finishing with the composition of the medium for the selection and regeneration of transformants, and reflects the number of stable independent transformants on conversion to the initial number of the inoculated explants.

The total number of the transformants was calculated after four monthly passages on the RT medium and one passage on the M medium complemented with cefotaxime and kanamycin. The data are presented in Fig. 6. From the diagram it is seen that from group A-1 to group A-4 the effectiveness of the transformation smoothly increases, and then on transition to A-5 it slightly decreases. The latter, most likely, is associated with lowering of the frequency of the transient GUS expression in this group, this, in its turn, lowering the frequency of formation of transgenic tissues. Another probable reason is lowering of the morpohogenetic potential in the case of long-term cultivation on hormone-free CC medium. However, the characteristic in any case is higher than in control group B. The average figure for groups A-1 — A-5 is 1.72%, this being almost two times higher than the control variant. Therefore, the stagewise co-cultivation increases the frequency of the regeneration of transgenic shoots.

Example 5. The influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the ratio of direct transformants and transformants produced via callus stage

It is known that the ratio of direct transformants and transformants produced via the callus stage depends mainly on the genotypic particulars of the plant (Masrcotrigiano et al. 1987; Suttter et al. 1997; Morozova, 2002). The developed method of genetic transformation of garden strawberry with the modified protocol of the preparation, inoculation and cultivation of explants is characterized by a higher proportion of direct transformants (Table 3).

Table 3. The influence of stagewise co-cultivation with *Agrobacterium thumefaciens* on the ratio of direct transformants and transformants produced via callus stage

Protocol	Variety	Frequency of trans-formation, %	Proportion of direct transformants, %
According to the improved method of stagewise co-cultivation (A)	Feyerverk	3.7	87.5
	Selekta	8.9	93.8
According to the known method (James et al. (1990) and Nehra et al. (1990) (B)	Feyerverk	1.0	28.3
	Selekta	1.8	42.0

Example 6. Data of the PCR analysis of transgenic plants of garden strawberry of Feyerverk variety, produced with the help of binary vector pBIThau35

Schestibratov et al. (2002), using an improved method of genetic transformation, produced transgenic plants of garden strawberry with the thaumatin genome. For studying T-DNA incorporation into the genome of kanamycin-resistant lines produced as a result of genetic transformations through the agency of the binary vector pBIThau35, a PCR analysis of samples of the total DNA was carried out. Since this vector provides transfer into the nucleus genome of two genes *nptII* and *thauII*, the introduction of T-DNA was analyzed separately for each sequence.

All the 23 independent transgenic lines are produced in stagewise selection on kanamycin in the concentration of 50 and 25 mg/l respectively in the first and subsequent passages. Then the produced regenerates were rooted on a medium with 25 mg/l of kanamycin. The PCR analysis of the samples of the total DNA for the presence of the fragment in 742 b.p. of the gene *nptII* has shown that all the lines contain genomic insert of the sequence under study. The results of the PCR analysis are presented in Table 4.

An analysis of the same samples of the total DNA with the use of a pair of primers to the sequence of the gene *thauII* has shown revealed that not all *nptII*-positive lines contain the insert of the gene of thaumatin II. PCR analysis has shown that the amplified fragment having the size of 878 b.p. was present in 18 out of 23 analyzed samples. The transgenic lines Clone 6, Clone 9, Clone 13, Clone 17 and Clone 20, in spite of the presence of the functioning insert of the gene *nptII*, did not contain the sequence of thaumatin II. The results of the PCR analysis are presented in Table 4.

Example 7. The data of Western-blot analysis of transgenic plants of garden strawberry of the Feyerverk variety, produced with the aid of binary vector pBITau35

The functionality of the introduced expression cassette *34S-thaumatin-3'nos* was analyzed by Western-blotting. Protein immunodetection in the vegetative tissues (leaves) has shown that the expression cassette is functioning and protein is synthesized in 15 out of 18 *thauII*-positive independent lines. In the transgenic lines Clone 2, Clone 4 and Clone 12 thaumatin is not expressed. The reason accounting for this fact may be defective insertion of the cassette *35S-thaumatin-3'nos* or endogenous suppression by homologous sequences. In the leaves of non-transgenic lines, as well as in the lines Clone 6, Clone 9, Clone 13, Clone 17 and Clone 20, without the insert *35S-thoumatin-3'nos* the presence of thaumatin II is not detected. The collective results of the carried out Western-blot analysis in the leaves of the transgenic plants are presented in Table 4,.

The immunological analysis of the thaumatin expression in the fruits of the transgenic plants has also confirmed the presence of protein (Table 4). The data correlate with the expression of thaumatin in the leaves.

Table 4. The results of PCR and Western-blot analyses of transgenic lines of garden strawberry (clones 1—23 and of the Feyerverk variety

Type of plant	PCR analysis for *nptII* gene	PCR analysis for *thauII* gene	Immunodetection of thaumatin in leaves	Immunodetection of thaumatin in fruits
Clone 1	+	+	+	+
Clone 2	+	+	—	—
Clone 3	+	+	+	+
Clone 4	+	+	—	—
Clone 5	+	+	+	+
Clone 6	+	—	—	—
Clone 7	+	+	+	+
Clone 8	+	+	+	+
Clone 9	+	—	—	—
Clone 10	+	+	+	+
Clone 11	+	+	+	+
Clone 12	+	+	—	—
Clone 13	+	—	—	—
Clone 14	+	+	+	+
Clone 15	+	+	+	+
Clone 16	+	+	+	+
Clone 17	+	—	—	+
Clone 18	+	+	+	+
Clone 19	+	+	+	+
Clone 20	+	—	—	—
Clone 21	+	+	+	+
Clone 22	+	+	+	+
Clone 23	+	+	+	+
Feyerverk	—	—	—	—

Example 8. The data of organoleptic analysis of the fruits of garden strawberry of Feyerverk variety

Tastings were carried out on the yields of garden strawberry for two years of cultivation on site for testing genetically improved plants on the territory of the VNIISPK (at Orel). The results of the tastings of the first year are presented in Table 5. The limited number of the first yield berries did not allow carrying out statistically reliable organoleptic analysis. Nevertheless, the average points evaluating the sweetness of the fruits, support a change in the taste properties of fruits of some transgenic lines. The sweetness of the fruit pulp was evaluated in terms of the five-point system, proceeding from the Feyerverk variety characteristic, according to which the taste of the mid-season fruits of the first yield is evaluated on an average by 4 points.

Table 5. Organoleptic analysis of sweetness of garden strawberry fruits of the first-year yield

Tasting 1		Tasting 2		Tasting 3	
Type of plant	Average score	Type of plant	Average score	Type of plant	Average score
Clone 3	4.1	Clone 1	4.0	Clone 3	4.0
Clone 12	3.9	Clone 17	3.8	Clone 6	3.8
Clone 11	4.0	Clone 16	4.0	Clone 7	4.0
Clone13	4.0	Feyerverk	3.9	Clone 13	4.0
Clone 22	4.2	Clone 21	4.0	Clone 8	3.9
Feyerverk	4.0	Clone 12	3.9	Clone 12	3.9

Pairwise comparison of the sweetness of berries with the thaumatin expression with control fruits has shown that in all the cases the scores of the experimental samples either excel the control ones (6 out of 9 pairs) or are equal to them (3 out of 9 pairs). Maximum exceedence of the sweetness of fruits with the thaumatin expression is 0.2 point. Such difference is registered in four pairs: Clone 3/Clone12; Clone 22/Feyerverk; Clone 1/Clone 17; Clone 3/Clone 6. In two cases the difference was 0.1 point: Clone 16/Feyerverk; Clone 21/Clone 12. Taking into account, in the first place, that for the Feyerverk variety the maximum score of the berries taste higher than 4 points is not typical, and , in the second place, the maximum score used in the selection practice is 4.5, the average taste score for Clone 22 exceeds appreciably the original variety characteristics.

The second-year yield proved to be more abundant and allowed carrying out statistically reliable organoleptic analysis of the fruits. The data are presented in Table 6.

Two tastings of the second-year yield have shown a reliable difference of five lines with the thaumatin expression out of the six selected ones from the control non-transgenic plants and lines without the thaumatin expression, except for the pair 3/13. The tasting scores for the second-year yield on an average are 0.2 point higher than those of the tasting of the first-year yield. This may be due to more favorable weather conditions contributing to the accumulation of sugars.

Table 6. Organoleptic analysis of sweetness of the second-year yield fruits of transgenic garden strawberry and of the Feyerverk variety

Tasting 1		Tasting 2	
Type of plant	Average score	Type of plant	Average score
Clone 3	4±0.03	Clone 16	4.2±0.03
Clone 12	4±0.09	Feyerverk	4.1±0.02
Clone 1	4.5±0.03	Clone 22	4.4±0.02
Clone 13	4.1±0.04	Feyerverk	4.2±0.12
Clone 8	4.3±0.09	Clone 21	4.3±0.07
Clone 6	4±0.12	Clone 6	4.1±0.05

Example 9. The influence of thaumatin II expression on the resistance of transgenic lines of garden strawberry of the Feyerverk variety to *Botrytis cinerea*

Basing on the results of CPR analysis and Western blotting, from the transgenic lines of strawberry produced by an improved method (Schestibratov et al. 2002) three clones were selected with the thaumatin expression level of at least 0.2 μg per mg of total protein, namely, Clone3, Clone 7 and Clone 8, and also one clone without the expression (Clone 17). Leaves from wild-type plants, from the lines without thaumatin expression (Clone 17) and from the transgenic line Clone GUS, produced with the use of the vector construct pBI121 were used as negative control.

Preliminary experiments have shown that the results of analyses are strongly influenced not only by the age of the plant tissue and the condition of the *Botrytis cinerea* inoculum, but also by the conditions under which the infected leaf disks are incubated. In the present case the key factor is the humidity of the chamber and of the filter supports. This factor affected not so much the character of the infection as the rate of colonization of leaf disks. Visual evaluation of the pathogen development was carried out after definite time intervals, for this reason humidity produced decisive influence on the results of comparison. Visual evaluation of necrosis, carried out on the sixth day after the inoculation, has given the following results: the mean area of affection was 27.5±12.9% for the Clone GUS, while on the disks of Clone 7 necrotic zones are not formed yet. The data about this and other pairs are presented in Table 7.

The data about the extent of sporogenesis, presented in Table 10 were registered on the ninth day after the inoculation (in contradistinction to the data about necrosis), since conidiofores are formed on mycelium with a delay of several days after the appearance of necrotic zones on leaf disks. Average scores of necrosis for example, for the

Clone 7/Clone GUS pair were 0.13±0.06/0.47±0.21 for the experimental and control variants, respectively.

Therefore, the data about the necrosis of leaf disks confirm statistically reliable differences not only within the pairs being compared but also between all the transgenic lines with the thaumatin expression on the one hand and the control plants on the other hand. The second criterion used by us for evaluating the resistance of plants to the infection caused by *Botrytis cinerea* also supports reliable differences within the pairs being compared, with the sporogenesis on the leaf disks of plants with the thaumatin expression having been substantially inhibited.

Table 7. The results of test for the resistance of leaf disks of transgenic garden strawberry and of the Feyerverk variety to *Botrytis cinerea*

Clone	Thaumatin accumulation level, μg/kg of total protein	Mean area of necrosis, %	Mean score of sporogenesis	Resistance enhancement index
Clone 7	1.5	0	0.13±0.06	3.6
Clone GUS	0	27.5±12.9	0.47±0.21	—
Clone 8	1	0.7±0.3	0.07±0.06	3.0
Feyerverk	0	5.7±5.5	0.21±0.11	—
Clone 3	0.2	1±0.8	0.13±0.06	2.8
Clone 17	0	19.7±6.1	0.37±0.11	—

Example 10. Field testing data of transgenic plants

Field testing of transgenic plants was carried out on the certified site for field testing of transgenic plants, created on the basis of the VNIISPK quarantine garden (at Orel). The vegetative and generative activities of field plants were evaluated according to four main criteria: the number of rosettes in a shrub; the average height of the shrub; the average number of peduncles; and the weight of ripe first-yield berries. After two years of growth in open ground conditions, the major part of transgenic lines did not display undesirable phenotypic variations (Table 8). However, several lines (Clone 9, Clone 14, Clone 15, Clone 128, Clone 19, Clone20, Clone 23) differed in the main characteristics from control non-transgenic plants and from the majority of other transgenic strawberry plants. Lines with somaclonal variations were visually detected mainly by the height of the shrub and by the yield (by the weight of the ripe first-yield berries). Eventually, an analysis for correspondence with the prototype has shown that 16 transgenic lines out of 23 in terms of the main characteristics of the vegetative and generative activities correspond to the Fey-

erverk variety. Therefore, the optimized method of genetic transformation of garden strawberry, described in the present invention, is characterized by an approximately 70% effectiveness of producing plants without somaclonal variations.

Table 8. The results of field testing of transgenic plants of garden strawberry and of the Feyerverk variety: evaluation of the vegetative and generative activities

Type of plant	Number of rosettes in a shrub	Average height of shrub, cm	Average number of peduncles	Weight of first-yield berries, g	Somaclonal variations
Clone 1	2.3±0.3	23.5±4.9	7.1±3.1	255±52	—
Clone 2	3.7±1.3	22.5±1.7	5.8±2	102±15	—
Clone 3	3.2±0.8	24.7±5.4	5±2.6	120±22	—
Clone 4	3.5±0.9	25.5±2.8	5±0.7	210±44	—
Clone 5	3.4±0.7	27±8.9	5.9±2.3	285±35	—
Clone 6	3.9±1.6	22.2±0.9	5.3±1.5	289±56	—
Clone 7	3.5±1	26.2±2.8	6.5±1.8	209±36	—
Clone 8	3.8±1.1	28.5±12	5.8±0.4	159±41\	—
Clone 9	3.3±1.3	19.8±1.9	2.3±1.7	43±13	+
Clone 10	3.5±0.8	21.5±.5	4.9±0.5	250±23	—
Cone 11	3.3±0.3	21±3	4.4±0.7	198±17	—
Clone 12	3.5±0.7	24.5±1.1	4.9±2.4	167±18	—
Clone 13	3.2±1.6	22.3±2.3	3.3±1.3	198±23	—
Clone 14	2.3±0.9	6.9±3.1	1.7±1.2	0	+
Clone 15	2.5±0.7	14.3±1.2	1.8±0.3	0	+
Clone 16	3.2±1	22.9±4.2	3.2±1.1	302±98	—
Clone 17	3.7±0.3	25.1±1.9	4.1±1	277±63	—
Clone 18	2.4±1	10.1±2.5	2..7±1.5	78±14	+
Clone 19	2.2±0.3	15.3±1.1	2.1±0.4	35±15	+
Clone 20	3.6±0.9	12.7±0.9	1.2±1.2	0	+
Clone 21	3.5±0.5	24.8±1.3	6.1±1.4	319±78	—
Clone 22	3.7±1.5	24.5±2	4.4±2.1	288±58	—
Clone 23	2.2±0.8	11.3±2.5	1.5±0.5	83±26	+
Feyerverk	3.2±0.7	24.2±2.9	4.8±1	266±35	—

Example 11. The influence of stagewise co-cultivation with Agrobacterium thumefaciens on transient GUS expression in tissues of apple explants

In the genetic transformation of apple of the Melba variety with the use of the improved protocol in the stage of transient GUS expression a higher frequency thereof was observed in groups A-3 and A-4 of variant A (as against control variant B). The results of the analysis are presented in Fig. 7. Similarly to the case with the garden strawberry, the frequency of the GUS expression was evaluated on conversion to the number of sections

which have gone interaction with agrobacteria, rather than on the number of explants. Apart from an increase of the GUS expression frequency, a change in the intensity of the GUS activity was observed. In variant A concurrently with the growth of the frequency, there took place an increase of the GUS staining intensity of explants after incubation in X-Gluc solution.

Example 12. Data of CPR and Western-blotting analyses of transgenic plants of apple, produced with the aid of binary vector pBIThau35

By the improved method of genetic transformation there were produced 2 independent transgenic lines of apple. The obtained regenerates were rooted on a medium with 25 mg/l of kanamycin. The PCR analysis of samples of the total DNA for the presence of a fragment having 742 b.p. of the gene *nptII* and of a fragment of the gene *thauII* having a size of 878 b.p. has shown that all the lines contain genomic inserts of two sequences (Table 9). The immunodetection of protein in the vegetative tissues (leaves) of plants in vitro and in vivo has shown that the expression cassette is functioning and protein is formed in both lines (Table 9).

Table 9. Results of PCR and Western-blotting analyses of transgenic lines of apple of Melba variety

Type of plant	PCR analysis for *nptII* gene	PCR analysis for *thauII* gene	Immunodetection of thaumatin in leaves in vitro	Immunodetection of thaumatin in leaves in vivo
Clone M-I-1	+	+	+	+
Clone M-I-2	+	+	+	+
Melba	—	—	—	—

Example 13. Data of CPR analysis of transgenic plants of Selekta variety, produced with the aid of binary vector pBIThau35

Using the improved method of genetic transformation, there were produced 15 independent transgenic lines. The obtained regenerants were rooted on a medium with 25 mg/l of kanamycin. The PCR analysis of samples of the total DNA for the presence of a fragment having 742 b.p. of the gene *nptII* has shown that all the lines contain a genomic insert of the sequence being studied. The results of the PCR analysis are presented in Table 10.

An analysis of the same samples of the total DNA with the use of a pair of primers to the sequence of the gene *thauII* has revealed, that not all *nptII*-positive lines contain the insert of the gene of thaumatin II. The PCR analysis has shown that the fragment being

amplified, having a size of 878 b.p., was present in 13 out of 15 samples being analyzed. Transgenic lines Clone S4, Clone S9, in spite of the presence of the functioning insert of the gene *nptII*, did not contain the sequence of thaumatin II. The results of the PCR analysis are presented in Table 10.

Example 14. Data of Western-blot analysis of transgenic plants of Selekta variety, produced with the aid of binary vector pBIThau35

The immunodetection of protein in the vegetative tissues (leaves) has shown that the expression cassette is functioning and protein is synthesized in all (13) of the *thauII*-positive independent lines. In the leaves of non-transgenic lines, like in the lines Clone S4, Clone S9 without the insert *35S-thaumatin-3'nos*, the presence of thaumatin II is not detected. The results of the carried out Western-blotting analysis are presented in Table 10.

Table 10. Results of PCR and Western-blotting analyses of transgenic lines of garden strawberry (clones S1—S15) and of Selekta variety

Type of plant	PCR analysis for gene *nptII*	PCR analysis for gene *thauII*	Immunodetection of thaumatin in leaves	Immunodetection of thaumatin in fruits
Clone S1	+	+	+	+
Clone S2	+	+	+	+
Clone S3	+	+	+	+
Clone S4	+	—	—	—
Clone S5	+	+	+	+
Clone S6	+	+	+	+
Clone S7	+	+	+	+
Clone S8	+	+	+	+
Clone S9	+	—	—	—
Clone S10	+	+	+	+
Clone S11	+	+	+	+
Clone S12	+	+	+	+
Clone S12	+	+	+	+
Clone S13	+	+	+	+
Clone S14	+	+	+	+
Clone S15	+	+	+	+
Selekta	—	—	—	—

The immunological analysis of the thaumatin expression in the fruits of transgenic lines has also confirmed the presence of protein. The data correlate with the thaumatin expression in leaves.

Table 11. Results of field testing of transgenic plants of garden strawberry (lines S1—S15) and of Selekta variety: evaluation of vegetative and generative activities

Type of plant	Average height of shrub, cm	Average number of peduncles	Average weight of a berry, g	Somaclonal variations
Clone S1	21.5±2.3	3.3±1.2		—
Clone S2	18±1.9	3±1.3		—
Clone S3	21±4	2.7±1.6		—
Clone S4	23.5±2.8	2.3±1.2		—
Clone S5	21±4.9	2±0.6		—
Clone S6	20.6±1.9	1.5±1		—
Clone S7	22.2±2.8	2.3±0.8		—
Clone S8	18.5±2	2.8±0.8		—
Clone S9	14.8±1	3.2±2		+
Clone S10	11.5±2.5	0.7±0.5		+
Clone S11	20±3	0.3±0.1		+
Clone S12	21.5±2.1	1.5±0.8		—
Clone S13	12.3±1.3	3.7±2		+
Clone S14	10.7±3	2.2±1		+
Clone S15	19.4±4.2	1.7±1		—
Selekta	20.2±1.8	2±0.8		—

Example 15. Field testing data of transgenic plants of Selekta variety

The vegetative and generative activities of field plants were evaluated according to the following main criteria: the average height of the shrub; the average number of peduncles; and the average weight of the berry. The analysis for the correspondence to the variety type has shown that 10 out of 15 transgenic lines in terms of the main characteristics of the vegetative and generative activities correspond to the Selekta variety. The lines with somaclonal variations were detected visually mainly by the height of the shrub, the number of peduncles, and the average weight of the berries (Table 11). Thus, the optimized method of the genetic transformation of garden strawberry, described in the present invention, is characterized by the effectiveness of producing plants without somaclonal variations equal to 66.7%, this being in good correlation with the data on the Feyerverk variety.

Example 16. Data of organoleptic analysis of fruits of transgenic lines of garden strawberry of Selekta variety

Pairwise comparison of the sweetness of the berries with the thaumatin expression with the control fruits has demonstrated that the characteristics of the experimental samples either excel or are equal to the control ones. Tasting has shown reliable enhancement of the sweetness in two lines (Clones S2 and S3) out of five with the thaumatin expres-

sion, selected for the analysis (Table 12). Maximum exceedence of the sweetness of fruits with the thaumatin expression is 0.3 point.

Table 12. Organoleptic analysis of sweetness of fruits of transgenic lines of garden strawberry and of Selekta variety

Tasting I		Tasting 2	
Type of plant	Average score	Type of plant	Average score
Clone S1	4±0.03	Clone S4	4±0.07
Clone S2	4.3±0.05	Clone S6	4±0.13
Clone S3	4.2±0.05	Clone S10	4±0.08
Selekta	4±0.07	Selekta	4±0.14

Industrial Applicability

The improved method of the preparation, inoculation and co-cultivation of explants has excelled all the effectiveness characteristics of the method of genetic transformation.. The present method is characterized by a low frequency of the necrosis of explants, an enhanced frequency of transient expression, an enhanced frequency of the transformation of transgenic tissues, an enhanced frequency of the regeneration of transgenic shoots, a higher proportion of direct transformants, a low frequency of somaclonal variations.

The method of genetic transformation, described in the present invention, has made it possible to produce a sufficient number of transgenic lines of garden strawberry of the Feyerverk and Selekta varieties, and draw confirmation of the effectiveness of the method in the genetic transformation of apple of the Melba variety. In the process of laboratory and field testing of the Feyerverk and Selekta varieties, success was made in selecting a number of lines with pronounced improved agronomic characteristics (improved taste of berries, enhanced resistance to grey mildew). Moreover these lines did not feature undesirable phenotypic variations which often originate in the *in vitro* regeneration of garden strawberry via the callus stage.

The improved method of the transformation provides an ample opportunity fort selecting genetic material for producing phytopathogen-resistant plants, plants oriented to the synthesis of proteins useful in pharmacology.

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